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(54) Title: METHOD FOR GENERATING INFLUENZA A VIRUSES BEARING ATTENUATING MUTATIONS IN INTERNAL PROTEIN GENES

(57) Abstract

A vaccine donor strain of influenza A virus is produced that is attenuated due to one or more mutations in an internal protein gene. The attenuating mutations are introduced into an internal protein gene and transcripts are transfected into cells infected with a helper virus capable of rescuing the gene encoded by the transfected nucleic acid. The helper virus is usually host range-restricted and incapable of growth on mammalian cells, whereas helper virus that incorporates the transfected gene can thereafter grow on mammalian cells. Vaccines can be produced by co-infecting cultured cells with the vaccine donor strain and a wild-type strain of influenza A. Attenuated reassortants containing the hemagglutinin and/or neuraminidase genes of the wild-type strain are then used as live influenza vaccines.

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METHOD FOR GENERATING INFLUENZA A VIRUSES BEARING ATTENUATING MUTATIONS IN INTERNAL PROTEIN GENES

BACKGROUND OF THE INVENTION

The present invention relates to methods for producing an attenuated vaccine donor strain of influenza virus. More particularly, a helper virus is used to rescue influenza internal protein genes which have attenuating mutations to produce the attenuated vaccine donor strain. The vaccine donor strain is then used to transfer its attenuating gene to a wild-type epidemic strain, producing a live attenuated vaccine virus that expresses the surface antigens of the epidemic strain.

The development of protective immunity in a host against viruses is often determined by the host's ability to mount an effective cellular and/or humoral immune response against epitopes on the viral surface. For influenza A virus, the protective immune response targets the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Antibodies directed against HA or NA can protect against virus challenge (Askonas et al., "The immune response to influenza viruses and the problem of protection against infection," Basic and Applied Influenza Research, CRC Press, Boca Raton, Florida (1982)). Accordingly, an effective influenza A vaccine must contain antigenic determinants corresponding to the HA and NA proteins of newly emerged pidemic strains of wild-type (wt) influenza A viruses. Becaus each new epidemic strain diff rs in its surfac proteins, new vaccin s ar requir d for each new influenza epidemic.

Influenza A viruses contain a segmented genome that is composed f negative strand RNA, i.e., RNA whose p larity is the opposite of that of messenger RNA (mRNA). The genome contains ten protein-coding genes, each of which has been mapped to one of the eight discrete RNA segments. Viral proteins include the HA and NA surface glycoproteins, a nucleoprotein, two non-structural proteins, three polymerase polypeptides, and the structural proteins M1 and M2. During replication, the genome is packaged with the nucleoprotein into a helical nucleocapsid, which itself is surrounded by a lipid envelope containing the HA and NA proteins. The polymerase proteins are located inside the virion where they are complexed with the genomic RNA. As is typical of viruses with RNA genomes, the less critical regions of the viral genes are subject to genetic drift, so that sequence variation is commonly observed among virus isolates. Regions of the proteins that are critical to protein function are highly conserved.

Individual segments of the influenza virus genome can reassort when cells are co-infected with two different strains of the virus. In this fashion, genes of one strain of influenza virus can be recovered into another strain.

Vaccine viruses may be live or inactivated. Inactivated vaccines include inactivated whole viruses or subunit virus vaccines. Subunit vaccines containing the HA and NA surface antigens are available for influenza virus. These subunit vaccines are less toxic but also are less antigenic than inactivated whole virus vaccines (Parkman et al., J. Infect. Dis. 146:S722-730 (1977)). They cannot replicate in the inoculated host and therefore stimulate an immune response that is different from that induced by infection.

The most common mode of inactivating whole virus vaccines, formalin inactivation, is less than satisfactory in many ways. For example, parenteral administration of formalin-inactivated virus provides only w ak immunity in the upper respirat ry tract, a maj r site f infecti n f r influenza virus. It has been reported that individuals

vaccinated with a formalin-inactivated measles vaccine lost their resistance to viral infection and developed atypical illness upon subsequent measles virus infection (Nader et al., J. Pediatr. 72:22-28 (1968)). In another instance, a formalin-inactivated respiratory syncytial virus (RSV) vaccine induced serum-neutralizing antibody, but did not protect against infection. Diseases such as Guillain-Barre syndrome have been associated with the inactivated influenza A "swine" flu virus vaccine (Schonberger et al. Ann. Neurol. 9(suppl.):31-38 (1981)). In general, when compared with live virus vaccines, inactivated vaccines have reduced cytotoxic T cell responses and increased incidence of delayed hypersensitivity reactions, and sometimes are associated with atypical illnesses.

The use of a live attenuated vaccine can provide the advantage of efficiently immunizing entire populations because the vaccine virus can spread from the vaccinee to unvaccinated hosts. Live attenuated influenza A virus vaccines are currently produced by transferring attenuating genes from a donor virus to new epidemic variants of influenza A virus, with the selection of reassortant viruses that possess the two surface glycoproteins of the epidemic virus (Murphy, Infect. Dis. Clin. Pract. 2:174-181 (1992)). Selection involves exposing the reassortant virus to antisera directed against the HA or NA of the donor virus. The recipient epidemic strain thus retains its surface antigens yet becomes an attenuated vaccine capable of eliciting the required protective antibodies.

Attenuated influenza donor viruses have been produced by the passage of virus at low temperature or by chemical mutagenesis. Vaccine donor strains can also be produced by rescuing synthetic HA or NA RNA transcribed from a cDNA copy of the gene. (Luytjes et al., Cell 59:1107-1113 (1989); Enami and Palese, J. Virol. 65:2711-2713 (1991); Seong and Brownl , Virology 186:247-260 (1992)).

An essential safety feature for a vaccine donor virus is that the attenuation be genetically stable. If the disabled internal protein of a live vaccine reverts to wild-

type, the vaccine itself will be as virulent as the original epidemic strain. Temp rature-s nsitive influenza vaccin s have been evaluated, but have exhibited incomplete attenuation or have tended to lose their attenuated phenotype during replication. Even vaccines containing two temperature-sensitive (ts) mutations have reverted to a virulent form following replication in vivo (Tolpin et al., Virology 112: 505-517 (1981)).

Avian influenza A virus single-gene reassortant (SGR) viruses have been used previously as vaccines. Clements et al., J. Clin. Micro. 30:655-662 (1992) have isolated and studied SGRs, each of which had acquired only one RNA segment from the parent influenza donor virus (A/Mallard/NY/78), and the remaining RNA segments from a human influenza A wild-type virus. In vitro, the PB2 SGR virus replicated efficiently in avian cells, e.g., primary duck kidney cells, but plaqued inefficiently in mammalian cells, such as Madin Darby Canine Kidney (MDCK) cells. In vivo, the PB2 SGR did replicate efficiently in avian hosts, but grew very poorly in primates and, hence, possessed the property of host-range restriction both *in vitro* and *in vivo*. The host-range restricted SGRs derived the PB2 gene from the A/Mallard/NY/78 parent, while the remaining genes were derived from the A/Los Angeles/2/87 wild-type parent (Clements et al., ibid.). Using this PB2 SGR as a vaccine, efforts were made to raise protective immunity to influenza A in human volunteers. However, this PB2 SGR virus proved to be so attenuated that it could not replicate at levels sufficient to trigger an immune response.

Another prototype attenuated virus is the cold-adapted variant derived from the wild-type strain A/Ann Arbor/6/60 (A/AA/6/60) (e.g., Cox et al., Virology 167:554-567 (1988)). This virus was derived by serial passage of the parent strain at successively lower temperatures until a variant emerged that was capable of efficient replication at 25°C. The ca mutant has three phenotypes, namely, temperature sensitivity, cold adaptation, and attenuation for the respiratory tract of animals. The PB1 and PB2 genes are independently responsible for the ts phenotype of the

influ nza A/AA/6/60 virus. Cox et al. cloned and sequenced six of the RNA s gments from A/AA/6/60 and the cold-adapted variant derived from it, i.e., ca A/AA/6/60. These comparisons revealed 24 nucleotide differences between the A/AA/6/60 ca mutant and its wild-type virus parent, which resulted in amino acid changes in all six of the internal viral proteins. It was found that the M2, PA, PB1, and PB2 proteins were likely to be involved in the phenotypic properties of the ca vaccine (Snyder et al., J. Virol. 62: 488-495 (1988)). The PB2 protein differed from the wild-type protein in predicted amino acid sequence only at position 265, which mutation was presumed to be responsible for the ts and attenuation phenotypes specified by the A/AA/6/60 ca PB2 gene. Live vaccines have been developed that contained the HA and NA from a new epidemic variant virus and five or six of their other genes from the cold-adapted (ca) A/AA/6/60 strain.

In addition to the PB2 mutation found in ca A/AA/6/60, other ts PB2 mutants are described in Shimizu et al., Virology 117:38-44 (1982), Shimizu et al., Virology 117:45-61 (1982), and Lawson et al., Virology 191:506-510 (1992).

While investigators have examined several approaches to producing a safe and effective live vaccine for influenza A, the results of currently available methods have been less than optimal. Primary drawbacks of present methods are the cumbersome selection procedures and the genetic instability of vaccines that rely on attenuating mutations. Consequently, an urgent need exists for a method that makes it possible to conveniently and quickly construct an influenza vaccine which contains phenotypic attributes of an emerging epidemic strain and has sufficient attenuating mutations in the genome so that genetic stability can be ensured. Quite surprisingly, the present invention fulfills these and other related needs.

Summary of the Invention

The present inv ntion provides methods and compositi ns for pr ducing attenuated vaccine d n r strains f influenza A virus and methods for using th donor strains to

influenza A/AA/6/60 virus. C x et al. cloned and sequenced six f the RNA segments from A/AA/6/60 and the cold-adapted variant derived from it, i.e., ca A/AA/6/60. These comparisons revealed 24 nucleotide differences between the A/AA/6/60 ca mutant and its wild-type virus parent, which resulted in amino acid changes in all six of the internal viral proteins. It was found that the M2, PA, PB1, and PB2 proteins were likely to be involved in the phenotypic properties of the ca vaccine (Snyder et al., J. Virol. 62: 488-495 (1988)). The PB2 protein differed from the wild-type protein in predicted amino acid sequence only at position 265, which mutation was presumed to be responsible for the ts and attenuation phenotypes specified by the A/AA/6/60 ca PB2 gene. Live vaccines have been developed that contained the HA and NA from a new epidemic variant virus and five or six of their other genes from the cold-adapted (ca) A/AA/6/60 strain.

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Summary of the Invention

The present invention provides methods and comp sitions for producing attenuated vaccine d nor strains of influenza A virus and methods for using the donor strains to

creat live att nuat d influenza vaccine strains. Th s methods includ the construction of influenza A viruses encoding modified internal proteins that cause attenuated viral growth in cultured cells or live hosts.

In an embodiment of the present invention, vaccine donor strains are created by using a helper virus to rescue human influenza A internal protein genes that contain attenuating mutations. The attenuating mutations may be introduced into the internal protein genes by in vitro mutagenesis. In certain embodiments the helper virus contains an internal protein gene that cannot support viral growth in mammalian cells. In this embodiment, non-mammalian cells are infected with the helper virus, and RNA encoding the attenuating internal protein gene is transfected into the infected cells. In some instances, the transfecting RNA is transcribed in vitro from DNA that encodes the attenuating gene, and the RNA packaged into ribonucleoprotein before transfection. Progeny from the transfected cells includes virus that has incorporated the transfected gene into virus particles, and that is specifically selected for in mammalian cell cultures, hence rescuing it into a live virus. Progeny containing the transfected gene exhibit attenuated growth in mammalian cells and serve as a vaccine donor strain.

One type of helper virus described in the present invention is capable of growth on non-mammalian but not on mammalian cells. One type of helper virus can grow on avian but not mammalian cells due to having a glutamic acid residue at position 627 in its polymerase basic protein 2 (PB2) (Subbarao, J. Virol. 67: 1761-1764 (1993), incorporated herein by reference). Vaccine donor strains created with a host range-restricted helper virus can be differentiated from the parent helper virus by their ability to grow in mammalian cells.

In a preferred embodiment of the present invention, the attenuating internal protein gene is polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), or the polymerase acidic prot in (PA). Mor than one attenuating mutation will typically be present in the internal protein

gene. In another mbodiment, more than one internal protein gene may sustain att nuating mutations.

In some embodiments of the invention the PB1, PB2, or PA gene will contain temperature-sensitive mutations that are responsible for the attenuation phenotypes. When the internal protein gene is PB2, the amino acid substitution causing temperature-sensitivity can include a substitution at amino acid positions 65, 100, 112, 174, 265, 298, 310, 386, 391, 417, 512, 556, or 658.

Also provided by the present invention is a method for manufacturing an influenza vaccine using the vaccine donor virus described herein. In this method, cultured cells are co-infected with the vaccine donor strain and a wild-type strain of influenza A virus. Vaccine virus is selected from the progeny of this co-infection by identifying and selecting reassortants that have incorporated the attenuating internal protein gene and either or both of the HA and NA genes of the wild-type strain.

In other embodiments the invention provides methods for stimulating the immune system of a human or an animal to induce protective immunity against wild-type influenza virus. The HA and/or NA of a wild-type virus, typically an emerging epidemic strain, is incorporated into an attenuated vaccine of the present invention, and the human or animal is inoculated with a dose sufficient to elicit an immune response.

The invention also includes attenuated reassortant viruses produced by the described methods.

Brief Description of the Drawings

Fig. 1 shows a diagram of the design of the AA wild-type PB2 plasmid, where T_3 is the truncated T_3 polymerase promoter sequence and HgaI is the restriction site at which the plasmid is cut prior to T_3 polymerase run-off transcription. The PB2 gene was amplifi d from virion RNA in two segments which were joined tog ther at a NcoI site and

cl ned between the PstI and Ec RI sites of pUC19 in a trim lecular ligation reaction.

Fig. 2 shows restriction digests with three different restriction enzymes run alongside uncut viral DNA on a 1% agarose gel, where parental virus is shown on top and the rescued virus is on the bottom.

Fig. 3 depicts a genotyping gel showing the origin of the RNA gene segments of the A/LA/2/87 AA wild-type PB2 (lane 2) and A/LA/2/87 AA mutant (mt) 265 PB2 (lane 4) transfectant viruses flanked by the virion RNAs of the A/AA/6/60 wild-type (lane 1) and A/AA/6/60 cold-adapted (ca) (lane 5) and the helper virus (lane 3). The 16 cm gels contained 2.6% acrylamide and 6.0 M urea. Electrophoresis was carried out for 16 h at 0°C and 100 V constant voltage. Viral gene segments were visualized after ammoniacal silver staining (Enami and Palese, J. Virol. 65:2711-2713 (1991); Boulikas and Hancock, J. Biochem. Biophys. Methods 5:219-228 (1981)). The gene assignments on the left indicate positions of the A/AA/6/60 virus genes and on the right indicate positions of the A/Mallard/78 X A/LA/2/87 PB2 SGR helper virus genes. Dots indicate the A/LA/2/87 genes present in the helper and transfectant viruses.

Fig. 4 shows the sequencing gels that demonstrate the sequence through nucleotides 805 to 849 of the AA PB2 gene. Arrows indicate position 821, which is an A in the A/AA/6/60 wild-type virus and a G in the A/AA/6/60 cold-adapted (ca) virus. The transfectant viruses show identical sequences as the respective "parent" viruses.

Fig. 5A and Fig. 5B show a comparison (in hamsters) of the level of replication of a PB2 transfectant virus bearing two (265 + 112) mutations in the PB2 gene with transfectant viruses with the wt PB2 gene or each mutation alone, where Fig. 5A shows level of replication in the nasal turbinates and Fig. 5B shows level of replication in the lungs.

Detailed Description of the Specific Embodiments

The present invention describes a general method wherein an internal protein gene of an influenza A helper virus is replaced by an internal protein gene synthesized *in vitro* from cDNA into which one or more attenuating mutations have been introduced by site-directed mutagenesis. With the present invention, single attenuating mutations or a combination of several such mutations can be introduced into a single internal protein gene, or into more than one internal protein gene. Live virus containing the attenuating internal protein gene can serve as a vaccine donor virus that is used to construct attenuated vaccine viruses containing the HA and/or NA genes of wild-type epidemic strains.

The helper virus preferably contains an internal protein gene that can be easily differentiated from the transfected (attenuating) internal protein gene. For example, the helper virus can be host-range restricted, and can encode an internal protein gene that cannot sustain growth on mammalian cells. In addition, the helper virus can encode HA or NA proteins whose epitopes differ from those of the epidemic strain.

After helper virus-infected cells have been transfected, progeny virus containing the rescued gene are plaque-purified and selected for the presence of the attenuating mutation. The selection conditions will depend on the specific phenotype of the attenuating mutation. For example, if the attenuating mutation specifies a ts phenotype, plaques are selected that can grow only at the permissive temperature. Variations of this strategy can be devised to accommodate the specific phenotype of the attenuating mutation. The selected virus then serves as a vaccine donor strain.

To produce a vaccine, cultured cells are co-infected with the vaccine donor strain and an epidemic wild-type strain. Reassortant viruses are harvested and individual plaques tested for the presence of phenotypes specified by the mutation, e.g., temperature-sensitivity. In addition or

alternatively, reassortants can be check d for the presenc of the wild-type HA and/or NA proteins by exposure to antiserum directed against the surface epitopes encoded by the donor virus. Reassortant viruses carrying the donor virus HA and/or NA are thereby eliminated. Progeny of individual viruses are obtained by plaque-purification.

Vaccines thus generated can be introduced by appropriate delivery route(s) into the animals or humans, preferably into the respiratory tract, to induce protective immunity against the epidemic strain.

Several methods are used for propagating influenza viruses of the present invention. For example, virus stocks may be plaque-purified, usually at least twice in primary cell cultures, which can include primary bovine or chick kidney cells. Plaque-purified virus can be propagated further in continuous cell lines or in primary cells such as primary rhesus monkey kidney cells (RMK), primary chick kidney cells (PCK), or in specific pathogen-free eggs. Cells are cultured on, e.g., 6-well or 24-well plastic tissue culture plates. Virus is typically inoculated at multiplicity of infection ("moi") of 0.1-10.0 moi and propagated for 1-5 days. To create reassortant virus strains, tissue culture cells are co-infected with two different plaque-purified viruses. For preparation of large quantities of plaque-purified virus, virus stock can be inoculated into the allantoic cavity of 9 to 11 day embryonated chicken eggs, and incubated for two days at 33°-40°C.

According to the present invention, RNA encoding one or more influenza A proteins is transfected into cultured cells. Techniques for transfecting influenza A virus RNA have been described previously (e.g., Luytjes et al. supra, and Enami et al., 1991 supra). In one method, primary chick kidney (PCK) cell monolayers are infected with the helper virus (for example, at a moi of 30-100 for 1 h for a wild-type gene at 37°C, or for 1 to 3 h at 32°C for a temperature-sensitive gene). The transcription reaction contains linearized plasmid, each of the deoxyribonucleotides (dNTPs), T3 RNA polymerase, and ribonucleoprotein prepared from virus

grown in the allantoic cavities of embryonated eggs (see, e.g., Parvin et al.; Enami et al., 1991). This mixture is incubated (e.g., 37°C for 55 min), during which RNA transcripts are produced and concurrently packaged into RNP particles. DNase is then added to the transcription mix to eliminate the plasmid, and the volume is transferred to the pre-infected, DEAE Dextran-treated PCK cells and incubated before being refed with appropriate medium, e.g., modified essential medium (MEM). Cultures are maintained (e.g., at 37°C for the wild-type gene and 32°C for the mutant gene) and are harvested at about 22 h later. Cells are pelleted and the virus-containing supernatant is plaqued on appropriate mammalian cells, e.g., Madin Darby canine kidney (MDCK) cells. Plaque progeny are subjected to at least one additional plaque passage (on, e.g., MDCK cells) and are then amplified in the allantoic cavities of embryonated eggs.

Influenza virus that has been attenuated as described herein is tested in well established in vitro and in vivo models to confirm adequate attenuation and immunogenicity for vaccine use. In in vitro assays the modified virus is tested for (i) the ability to react with antibodies directed at surface epitopes and (ii) the temperature sensitive phenotype or other in vitro marker of attenuation. Modified viruses are further tested as described below in animal models of influenza infection.

Testing for temperature-sensitivity in cultured cells can be accomplished essentially as described in Shimuzu et al., Virology 117:38-44 (1982), which is incorporated herein by reference. In one method, for example, aliquots of plaque-purified virus (0.1 ml) are inoculated onto cultured cells in multi-well plates, adsorbed for 1 h at room temperature, and each well overlaid with about 2 ml of 0.9% agarose in culture medium. Replica plates are incubated in 5% CO_2 at 34°C and at 40°C for 2 days. Virus harvested from each well is used to inoculate RMK or MDCK cells, which are incubated at 34°C or 40°C. Virus that grows only at the lower temperature are provisional mutants. To confirm the temperature sensitivity of these viruses, they are titrated by

TABLE 1

Wild-type PB2 Gene Sequenced	Ts Sequenced	Nucleotide Position	wt sequence	ts sequence	Amino acid Position	wt	ts
A/Udorn/307/72	UV257AA	221	GAA	GGA	65	Glu	Gly
	UV1466CA	325	AAT	GAT	100	Asn	Asp
	ICRC303AA	361	CCA	TCA	112	Pro	Ser
	SP374AB	361	CCA	TCA	112	Pro	Ser
	SP959CC	361	CCA	TCA	112	Pro	Ser
	SP37AA	548	GCC	GAC	174	Ala	Asp
	ICR1397AA	919	CTT	TTT	298	Leu	Phe
	ICR1439CB	956	ATA	ACA	310	Ile	Thr
	5FU623A	1183	GTG	ATG	386	Val	Met
	ICR1027CB	1198	GAG	AAG	391	Glu	Lys
	ICR348EA	1908	AAG	AAA	---	---	---
		1693	AAC	GAC	556	Asn	Asp
A/GL/0389/65	A/Ud/72ts-1	1999	TAC	CAC	658	Tyr	His
A/AA/6/60	A/AA/6/60 ca	821	AAC	AGC	265	Asn	Ser
A/WSN/33	A/WSN/33 ts	1276	GAC	AAC	417	Asp	Asn
A/FPV/Ros/34	A/FPV/Ros/34 ts	1561	CTC	ATC	512	Leu	Ile

plating serial dilutions at RMK or MDCK cells at both temperatures using an overlay of 0.8% agarose containing tissue culture medium. Plaques on MDCK cells are clearly visible but plaques on RMK cells are visualized by hemadsorption with 0.1% guinea pig red blood cells in phosphate-buffered saline (PBS) after removal of the overlay. Virus that exhibits a 40/34° plaque titer of 10^{-2} are considered ts mutants.

Cloning and sequencing the PB2 genes from a number of ts isolates has revealed ten specific amino acid positions at which substitutions result in a temperature-sensitive phenotype (Lawson et al., Virology 191:506-510 (1992), which is incorporated herein by reference). The nucleotide sequences for many of the mutant PB2 genes were compared with sequences of the wild-type strains from which the mutants were derived. In some cases where the parent strain was not available, the ts sequences were compared with non-PB2 mutants that were sister strains of the PB2 mutant strain. Three other ts mutations in PB2 have been reported. For all thirteen of the described ts mutants, single amino acid substitutions have been located within highly conserved regions of the PB2 gene. The following Table 1 depicts the sequence comparisons from Lawson et al.:

Each ts mutant has only a single amino acid substitution, suggesting that these substitutions cause the ts phenotypes of the viral isolates. All of the substitutions are located within the coding region of the gene. No insertions or deletions were observed, indicating that the PB2 protein is conserved in length.

Using this panel of ts mutations for PB2, a large number of combinations of mutations can be evaluated to identify the most satisfactorily mutated PB2 gene. The amino acid present at each ts locus can be substituted with other amino acids or the site of the mis-sense mutation manipulated by deletional or insertional mutagenesis. Ts mutants of PB1 and PA are identified using methods similar to those used for elucidating ts mutants of PB2.

Temperature-sensitive (ts) attenuating mutations are used in the present invention for developing genetically-engineered attenuated influenza A vaccines. Ts mutants are restricted in growth in the lower respiratory tract (37 - 39°C), but still can elicit protective immune responses because they replicate to high titers in the upper respiratory tract where temperatures are permissive (32-34°).

Since viruses containing single ts mutations are likely to be genetically unstable in vivo, various configurations of two or more of the ts mutations results in a PB2 gene that is satisfactorily attenuated, genetically stable, and immunogenic. Preferred combinations of two or more ts mutations are PB2-112 and PB2-265, PB2-265 and PB2-556, or PB2-265 and PB2-658, although other combinations of two or more ts mutations in the PB2 gene also provide genetic stability, adequate attenuation and protective immunogenicity, e.g., the use of three mutations in PB2, such as the combination of PB2-112, 265 and 658, or PB2-112, 265 and 556. Stability of the PB2 gene containing 1, 2, 3 or more ts mutations can also be enhanced by ts mutations in the PA gene. These PB2/PA mutations can be generated by reassorting gene segments from PB2 mutant transfectant viruses and ts PA mutants. Stability of the mutated PB2 gene can also be enhanced by combining both ts and regulatory mutations in the

same donor virus. An example of a suitable attenuating regulat ry mutation is the substitution of the 3' and 5' untranslated regions of influenza A with the analogous sequences taken from an influenza B RNA segment, as described in, for example, copending application of Palese et al., USSN No. 07/938,716, which is incorporated herein by reference. The 5' untranslated region plays a critical role during transcription, serving as a binding site for the viral RNA polymerase. Influenza B virus 5' sequences can support transcription by influenza A virus polymerase.

Rescuing artificially-introduced mutations such as the ones described herein provides a means for constructing an optimal live influenza vaccine. In general, the optimal donor strain for vaccine use must be satisfactorily attenuated, immunogenic, and genetically stable after being administered to seronegative individuals.

Recombinant influenza genes altered by site-specific mutagenesis ~~can be~~ rescued into live influenza virus. Means for rescuing recombinant influenza virus genes are described in Enami et al., Proc. Natl. Acad. Sci. 87:3802-3805 (1990), and Enami et al., J. Virology, 65:2711-2713 (1991), which are incorporated herein by reference. The procedure involves transfection of recombinant RNA into cells that are infected simultaneously with infectious helper virus. Some of the viral progeny from these cells will contain an RNA segment derived from the transfected gene.

The transfection of influenza virus genes is carried out with viral RNA that has been packaged into RNP particles. An efficient method for transferring recombinant RNA into live virus is as follows. First, conventional techniques are applied to introduce the desired mutations into a cDNA copy of the RNA segment encoding the target protein. For example, mutations can be introduced by using the Muta-gene phagemid in vitro mutagenesis kit available from BioRad Laboratories, Richmond, CA. In brief, the wild-type gene is cloned into the plasmid pTZ18U, and us d to transform DH5α F' cells (Life Technologies Inc., Gaithersburg, MD). Phagemid preparations are prepared as recommended by the manufacturer.

Oligonucl otides are designed f r mutagenesis by intr duction of an altered nucle tide at the desired position. The plasmid containing the genetically altered gene is then amplified.

To transfer the altered gene into a live influenza virus, the plasmid is transcribed in vitro to produce influenza virus RNA which is concurrently packaged into infectious RNP by influenza virus proteins added to the reaction mixture. Aliquots of this reaction mixture are transfected into cells using a DEAE-dextran RNP transfection protocol such as that described by Enami et al., Proc. Natl. Acad. Sci. USA 87: 3802-8305 (1990) or Enami et al., J. Virol. 65: 2711-2713 (1991). An advantage of using genetically-engineered donor viruses is that the mutations can be monitored during all phases of vaccine production and use.

Administration of virus suspensions to animals or humans are typically propagated in 9 to 11-day-old specific-pathogen-free eggs, and are then tested for the presence of adventitious agents. Infectivity titers are determined by plating serial dilutions into MDCK or equivalent cells. Vaccine strains are grown in either pathogen-free embryonated eggs or tissue culture. Virus is harvested and resuspended in a suitable carrier, e.g., water, 0.15 ml NaCl or the like, containing appropriate stabilizers such as sucrose, phosphates, glutamate, calcium, etc. If desired, the virus may then be lyophilized. The dose of the vaccine of the present invention which is administered to an individual is typically about 10^5-10^8 infectious units per vaccinee, usually in about 0.1-1.0 ml.

For vaccine use, the attenuated virus can be used directly in vaccine formulations, or lyophilized in individual or multiple dose containers. The suspended or lyophilized virus is provided as a pharmaceutical composition in a pharmaceutically acceptable carrier. Lyophilized virus will typically be maintained at about 4°C. For use as a vaccine, lyophilized virus is reconstituted in a stabilizing solution, e.g., saline or comprising SPG (Bovarnick et al., J. Bacteriol. 59: 509-522 (1950)), Mg^{++} and HEPES, with or without adjuvant.

Attenuated influenza vaccine virus of the present invention is administered to humans or animals in quantities and in a manner sufficient to elicit an immune response that is protective against subsequent infection by wt virus. Means of administration to a mucosal surface include aerosol, droplet, coarse spray, oral, topical or other routes. The most preferable means are those suitable for intranasal delivery.

The attenuated vaccine virus of the invention can be monitored in well established models for influenza vaccines, such as hamsters, mice, or squirrel monkeys. For example, squirrel monkeys housed in Horsfal isolation units are inoculated intratracheally with 10^7 $TCID_{50}$ of virus in a 0.5 ml inoculum. To monitor the course of infection, nasopharyngeal swab specimens are obtained daily for 10 days post-inoculation, and tracheal lavage fluids obtained on 2,4,6, and 8 days post-inoculation. Aliquots of these specimens are frozen for subsequent titration on MDCK cultures or in eggs. The mean duration and peak titer of virus shedding are determined for each monkey, and these parameters compared for monkeys that received either wild-type human influenza A virus or a reassortant virus. The use of hamsters to monitor efficacy of vaccine prototypes is described in detail in Example 6 below.

The following examples are offered by way of illustration, not by way of limitation.

Example 1

Site-Directed Mutagenesis of the Wild-Type A/AA/6/60 PB2 Gene

An amino acid substitution at position 265 associated with the attenuated and cold-adapted phenotype of ca A/AA/6/60 (Cox et al., Virology 167:554-567 (1988)) was created within the coding region of PB2 by changing an A to a G at position 821. The wild-type PB2 gene of the A/AA/6/60 virus from virion RNA was cloned in two cDNA segments (1-1248 and 1219-2341) that wer reverse transcribed with AMV reverse transcriptase (Lif Scienc s, Inc., St. Petersburg, FL) from a

viral RNA template. The complete cDNA "gene" was amplified using the polymerase chain reaction (PCR; described in Sambrook et al., Molecular Cloning, Ch. 14, which is incorporated herein by reference), and the resulting DNA segment inserted into the pUC19 cloning vector as shown in Fig. 1. The insert of the resultant plasmid, pT3AAwtPB2, was sequenced and the sequence compared with that of the A/AA/6/60 wild-type PB2 gene. This comparison indicated that no coding changes had occurred during the cloning steps. Before being used as a template in subsequent transcription reactions, plasmid DNA was digested overnight with HgaI and the overhanging single-stranded region was filled in with DNA polymerase Klenow fragment.

To insert a mutation at nucleotide 821 into the cloned wild-type PB2 gene, the procedure outlined for the Muta-gene phagemid in vitro mutagenesis kit was followed (BioRad Laboratories; Richmond, CA). As a first step, the insert from the plasmid pT3AAwtPB2 was cloned into pTZ18U and was used to transform DH5α F' cells. The oligonucleotide designed for mutagenesis was as follows: nt 812 5' CAGCCAGGAGCATAGTGA 3' [SEQ ID No. 1]. The recovered mutagenized A/AA/6/60 PB2 cDNA was sequenced and found to differ from the wild-type PB2 plasmid only at nt 821.

Example 2

Rescue of the Cloned A/AA/6/60 Wild-Type PB2 Gene Into the SGR Helper Virus

This Example demonstrates the efficacy of using a host range-restricted SGR as a helper virus to rescue modified influenza genes.

The helper virus used for this Example was the earlier-described avian-human influenza A virus PB2 SGR virus (with an avian PB2 gene) that replicates efficiently in avian tissue but poorly in mammalian cells. This SGR strain derives its PB2 gene from the avian influ nza A/Mallard/NY/6750/78 and its remaining genes fr m the human influenza A/Los

Ang 1 s/2/87 (A/LA/2/87) (H3N2) virus (Clements et al. J. Clin. Micro. 30:655-662 (1992)).

Wild-type PB2 viral RNA was transcribed in vitro from pT3AAwtPB2, and was rescued as follows. The RNA was packaged into RNP and transfected into primary chick kidney (PCK) cells pre-infected with the SGR helper virus. Transfections were done using DEAE Dextran in a modification of the technique of Luytjes et al. (Cell 59:1107-1113 (1989)). For the helper virus infection step, PCK monolayers in 35 mm culture dishes were infected at a multiplicity of infection of 30-100 for 1 h at 37°C. The transcription reaction (50μl) contained 2.5μg linearized and Klenow-filled plasmid, 1 mM each of dNTPs, 0.1M dithiothreitol, 150 units T3 polymerase, and 25-35 μl of ribonucleoprotein (RNP) prepared from the avian influenza virus A/Duck/Oklahoma/77 virus. After incubation at 37°C for 55 minutes, DNase was added for 5 minutes at 37°C followed by the addition of 100μl of PBS containing 1 mg/ml gelatin to the transcription mix, and 150 μl of the mixture transferred to the pre-infected, DEAE Dextran-treated PCK cells and incubated for 1 h at 37°C before being refed with 1 ml of minimal essential medium (MEM) containing 4% bovine serum albumin.

Cultures of transfected cells were maintained at 37°C until being harvested 22 h later. The cells were pelleted by centrifugation, and the supernatant plaqued onto Madin Darby canine kidney (MDCK) cells. Plaque progeny underwent one additional plaque passage on MDCK cells and then were amplified in the allantoic cavities of embryonated eggs.

Example 3

Rescue of the Synthetically-Mutated PB2 Gene Into the SGR Helper Virus

The feasibility of introducing ts mutations into PB2 was established by rescuing the PB2 gene of the A/AA/6/60 virus that had a single amino acid substitution introduced at position 265 by site-directed mutagenesis. The rescue procedure was similar to that described for the wild-type PB2

gene in Example 2, except for the following changes in incubation temperature. After monolayers of PCK cells were transfected, the first incubation was for 3 h at 32°C instead of 1 h at 37°C. After the DNase step, cells were maintained at 32°C instead of at 37° until virus harvest 22 h later.

Example 4

Identification of Rescued PB2 Genes

Restriction endonuclease analysis was used to identify the rescued PB2 genes. Following proteinase K digestion, RNA was extracted from the allantoic fluid cultures of each plaque progeny by extraction with phenol and chloroform (See Sambrook et al., supra). A 1 kilobase segment of the PB2 gene was amplified by PCR using a set of oligonucleotide primers designed for sequences conserved among the PB2 genes of A/AA/6/60, A/Duck/OK/77, and A/Mallard/NT/78 (nt 1110 (positive sense) 5' GAGTTCACAATGGTTGG 3' [SEQ ID No. 2] and nt 2000 (negative sense) 5' TTGTTGTAGTTGAATA 3' [SEQ ID No. 3]). Amplification of the gene segment from the A/Duck/OK/77 virus was inefficient. The PCR-amplified DNA was divided into four aliquots, three of which were digested for 1h at 37°C with the restriction endonucleases BstXI, Ksp632I, or HhaI. The uncut DNA and the digested samples were electrophoresed in a 1% agarose gel and the patterns of migration were compared with the patterns of control PB2 derived from the helper virus, the virus used to generate the RNP, and the plasmid. The pattern of digestion with the three enzymes was different for the three possible parental genes and allowed easy identification of the source of the PB2 gene in the putative transfectant viruses (Fig. 2).

Sequence analysis also was used to verify the source of rescued PB2 genes. The PB2 gene was amplified in two separate reactions as overlapping segments from viral RNA by PCR (nt 1-1240 and 1141-2341) using AMV reverse transcriptase to synthesize the first strand and conventional methods of PCR amplification. The nucleotide sequences of the PCR products were determined using the CircumVent thermal cycler dideoxy

DNA sequencing kit (New England Biolabs) with ^{35}S-dATP. The entire length of the gene was sequenced using 13 synthetic oligonucleotide primers. Fig. 4 compares the sequencing reactions for parent viruses and the rescued wild-type and ts mutants of PB2. The nucleotide differences are evident in Fig. 4.

Example 5

Temperature Sensitivity of AA Wild-Type and Mutant PB2 Transfectant Viruses

The efficiency of plaque formation at different temperatures of the two wild-type and one mutant PB2 transfectant viruses were compared with each other and with a variety of control viruses (Table 2). The permissive temperature was 32°C, and elevated temperatures tested were 38°, 39°, and 40°.

The A/LA/2/87 wild-type virus was included in the comparison because it most closely resembles the genotypes of the three transfectant viruses (12A3, 19A1, and 10A1), being identical to them except in the PB2 gene (Fig. 3). Two of the transfectants (12A3 and 19A1) derived their PB2 gene from wild-type A/AA/6/60 PB2 gene. The A/LA/2/87 6-2 ca reassortant virus was included because it contains two ts genes (PB1 and PB2) from the A/AA/6/60 ca donor virus and therefore represented a maximum level of growth inhibition at the non-permissive temperature that could be manifested by the A/LA/2/87 AA mt 265 PB2 transfectant virus.

The A/Korea/82 PB2 SGR virus, which contains the AA ca PB2 gene in a background of genes from a wild-type influenza A H3N2 virus, was also included in this set of comparisons. This SGR was included because its genetic composition is essentially equivalent to that of the PB2 mt transfectant. The A/Korea/82 SGR contains the AA ca PB2 gene (identical in coding sequence to the transfected mt PB2 gene) in an H3N2 background, hence it would be expected to be similar in its temperature-sensitivity to the mutant transfectant which bears the equivalent PB2 gene, also in an

H3N2 background. Growth properties of the two viruses were similar, except for a 1°C difference in their shut-off temperatures (Table 2).

The A/LA/2/87 and A/Korea/82 wild-type viruses replicated to similar titers at permissive and restrictive temperatures (Table 2). A marked restriction in replication at 39°C was exhibited by the 6-2 gene ca reassortant viruses that derive their HA and NA genes from the wild-type viruses A/LA/2/87 or A/Korea/82 and six internal genes from the A/AA/6/60 ca donor virus. The A/LA/2/87 AA wild-type PB2 transfectant viruses behaved like the wild-type viruses, i.e., with no difference in level of replication at 40°C, whereas the A/LA/2/87 AA mt 265 PB2 transfectant virus was markedly restricted at 40°C but not at 39°C. The A/LA/2/87 and A/Korea/82 ca reassortant viruses each derive two genes from the A/AA/6/60 ca virus that independently confer the ts phenotype (namely the PB1 and PB2 genes), while the A/LA/2/87 AA mt 265 PB2 transfectant virus has only the mutant ca-equivalent PB2 gene. This difference may explain the observation that the shut-off temperature for the latter was 1°C higher than that of the 6-2 ca reassortant viruses.

TABLE 2

COMPARISON OF THE ts PHENOTYPE OF PARENT, REASSORTANT AND TRANSFECTANT VIRUSES

Virus tested	log_{10} reduction in pfu/ml at indicated temperature compared with permissive temp (32°C)[a]			Shut-off temperature[b]
	38°C	39°C	40°C	
A/LA/2/87 wt	0.0±0.1[c]	0.0±0.1	0.0±0.1	≥41°C
A/LA/2/87 6-2 ca reassortant	0.7±0.7	5.1±1.7	6.5±0.3	39°C
A/LA/2/87 AA wt PB2 transfectant 12A3	0.0±0.1	0.5±0.1	0.9±0.1	≥41°C
A/LA/2/87 AA wt PB2 transfectant 19A1	0.2±0.1	0.5±0.1	1.2±0.5	≥41°C
A/LA/2/87 AA mt 265 PB2 transfectant 10A1	0.4±0.1	1.3±0.2	4.9±0.6	40°C
A/Korea/1/82 wt	0.0±0.2	0.2±0.2	0.5±0.3	≥41°C
A/Korea/1/82 6-2 ca reassortant	1.7±0.6	4.8±1.1	5.7±0.2	39°C
A/Korea/82 PB2 SGR	1.0±0.6	5.2±0.7	5.9±0.3	39°C

[a]Titers at 32°C ranged from 5.5 to 7.6 log_{10}.
[b]Shut-off temperatures are defined as the lowest restrictive temperature at which there is a ≥2 log reduction in titer from the titer at the permissive temperature.
[c]Titer reduction are expressed as mean log_{10} pfu/ml ± std error from three experiments.

Example 6

Mutant Transfectant Virus is Attenuated for the Upper and Lower Respiratory Tract

The level of replication of the A/LA/2/87 AA wild-type PB2 transfectant was compared to that of the A/LA/2/87 AA mutant 265 PB2 transfectant virus in the respiratory tract of hamsters. These two viruses differed in sequence by only one amino acid, and thus the effect of the single mutation on replication in vivo was directly assessed.

Anesthetized 5 week old female Golden Syrian hamsters received 10^5 $TCID_{50}$ of virus in 0.1 ml intranasally. Strains inoculated were virus recovered after transfection with either the synthetic PB2 gene derived from the A/AA/6/60 wild-type influenza A virus, or the A/AA/6/60 PB2 gene mutated in vitro at nucleotide 821. After days 1, 2 and 3 following virus administration, animals were sacrificed and lung and nasal tubinates areas were removed; a 5% (w/v) suspension of nasal turbinates and 10% (w/v) suspension of lung tissue was made and the virus titer in each was determined in MDCK tissue culture. The results are depicted in Table 3, in which the titers are reported as mean $\log_{10}$ $TCID_{50}/g \pm$ standard error.

Because the A/LA/2/87 AA mutant 265 PB2 transfectant virus had a shut-off temperature of 40°C, and the body temperature of hamsters is about 39°C, only a mild to moderate degree of attenuation of this virus was expected for hamsters. The A/LA/2/87 mutant 265 PB2 transfectant virus showed a modest restriction of replication compared with the A/LA/2/87 AA wild-type PB2 transfectant virus (Table 3). Both transfectant viruses were less restricted in replication than the 6-2 gene cold-adapted reassortant viruses evaluated previously (Murphy, Infect. Dis. Clin. Pract. 2:174-181 (1992)). Highly significant differences (student's t-test) were observed in virus titers achieved over the 3 days post-inoculation in the lungs ($p = 0.02$) and in the nasal turbinates ($p = 0.002$) of the 18 hamsters that r ceived the A/LA/2/87 AA mutant 265 PB2 and A/LA/2/87 AA wild-type PB2

transfectant viruses. This indicat d that the PB2 gene bearing a single mutation at the amino acid position 265 specified the attenuation phenotype in hamsters.

These results illustrate that the single amino acid substitution in the PB2 gene resulted in attenuation of the virus for the upper and lower respiratory tracts of hamsters, as evidenced by the reduction in amount of virus produced over the three days tested, compared with that of the transfectant virus bearing the wild-type PB2 gene. The mutation conferred only about a 10-fold reduction in replication in the lower respiratory tract and thus the single amino acid substitution present in the mutated PB2 virus did not sufficiently attenuate the virus for this site. However, using the methods of the present invention, the mutant PB2 gene served as a substrate for the introduction of additional attenuating mutations, as described below.

TABLE 3

THE A/LA/2/87 AA mt 265 PB2 TRANSFECTANT VIRUS IS ATTENUATED FOR THE UPPER AND LOWER RESPIRATORY TRACT OF HAMSTERS[a]

A/LA/2/87 virus administered	No of animals tested per day	Mean peak titer (log_{10} $TCID_{50}$/g of virus present in indicated tissue)					
		Nasal Turbinates			Lungs		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
AA mt PB2 transfectant 12A3	6	4.8±0.2	5.9±0.1	5.4±0.3	3.6±0.7	3.6±0.7	4.4±0.6
AA wt 265 PB2 transf ctant 10A1	6	4.2±0.3	5.1±0.2	4.9±0.1	2.3±0.4	2.7±0.5	3.1±0.6

[a]Anesthetized 5 wk old female Golden Syrian hamsters received $10^{5.0}$ $TCID_{50}$ of virus in 0.1 ml intranasally. After animals were sacrificed on days 1, 2 and 3, A 5% (w/v) suspension of nasal turbinates and 10% (w/v) suspension of lung tissue was made and the virus titer in each was determined in MDCK tissue culture. The titers are reported as mean log_{10} $TCID_{50}$/g ± std error.

Example 7

Immunogenicity of Influenza Virus Bearing an Attenuating PB2 Gene

The ability of the A/LA/2/87, AA wild-type, and mutant 265 PB2 transfectant viruses to induce an antibody response and protect animals from challenge with influenza A virus was determined. Anesthetized 5 week old female Golden Syrian hamsters were inoculated with 10^5 $TCID_{50}$ of virus in 0.1 ml intranasally and were challenged 31 days later with 10^5 $TCID_{50}$ of A/LA/2/87 wild-type virus in 0.1 ml intranasally. One day later, at the expected peak of replication of the A/LA/2/87 wild-type virus, the hamsters were sacrificed and a 5% (w/v) suspension of nasal turbinates and 10% (w/v) suspension of lung tissue was made and the virus titer in each was determined in MDCK tissue culture.

As shown in Table 4, despite its restricted replication in the lungs of hamsters, the A/LA/2/87 AA mt 265 PB2 transfectant virus was immunogenic and induced significant resistance to subsequent challenge with the A/LA/2/87 wild-type virus.

TABLE 4

THE A/LA/2/87 AA mt 265 PB2 TRANSFECTANT VIRUS IS IMMUNOGENIC AND PROTECTS HAMSTERS FROM wt VIRUS CHALLENGE[a]

Immunizing Virus	No. tested	Serum HAI antibody titer at day 28[b]	Mean titer (log_{10} $TCID_{50}$/g) of virus present in indicated tissue[c]	
			Nasal turbinates	Lungs
B/AA/1/86	7	≤3.0±0.2	6.0±0.1	3.7±0.7
A/LA/2/87 AA wt PB2 transfectant 12A3	6	7.6±0.2	2.6±0.6	≤1.5±0
A/LA/2/87 AA mt 265 PB2 transfectant	6	6.8±0.2	2.7±0.5	1.6±0.2

[a]Anesthetized 5 wk. old female Golden Syrian hamsters were immunized with $10^{5.0}$ $TCID_{50}$ of virus in 0.1 ml intranasally and were challenged 31 days later with $10^{5.0}$ $TCID_{50}$ of A/LA/2/87 wt virus in 0.1 ml intranasally. One day later, at the expected peak of replication of A/LA/2/87 wt virus, the hamsters were sacrificed and a 5% (w/v) suspension of nasal turbinates and 10% (w/v) suspension of lung tissue was made and the virus titer in each was determined in MDCK tissue culture.

[b]Serum hemagglutination-inhibiting (HAI) antibody titer measured against A/LA/2/87 wt virus and expressed as mean log_2 titer ± std error.

[c]Titers are expressed as mean log_{10} $TCID_{50}$/g ± std error.

Example 8

Generation f PB2 Transf ctant Viruses B aring Tw Site-Specific Temperature Sensitive Mutations

This Example describes introducing an additional mutation into the PB2 gene segment to generate a single attenuating influenza A virus PB2 gene segment that can be used alone or in combination with another attenuating gene segment as a vaccine donor strain.

The PB2 single gene reassortant (SGR) virus A/Mallard/NY/78 X A/Los Angeles/2/87 that derives its PB2 gene segment from the mallard influenza A virus and remaining gene segments from the human influenza A virus, as described in Example 2, supra, was used as the helper virus in transfection experiments. The avian influenza A/Duck/4/77 virus used for purifying ribonucleoprotein has been described (in Example 2). The generation of the A/LA/2/87 Ann Arbor (AA) wt PB2 transfectant virus and the A/LA/2/87 AA mt 265 transfectant virus by transfection rescue techniques is described in Examples 2-4. (The isolation and passage history of the A/Los Angeles/2/87 (A/LA/2/87) wt virus which was used as a challenge virus in testing the efficacy of the double mutant virus as a vaccine is described in Clements et al., supra, and the isolation and passage history of the influenza B/Ann Arbor/1/86 (B/AA/1/86) virus, used as a control for immunogenicity and challenge experiments, is described in Herlocher et al., "Genetics and Pathogenicity of Negative Strand Viruses," Kolakofsky and Mahy, eds., p. 387-401, Elsevier, Amsterdar 1989).)

Transfections were carried out in primary chick kidney cell monolayers prepared from kidneys of 3 to 5 day old specific-pathogen-free chicks as described in Enami et al., J. Virol. 65: 2711-2713 (1991). Transfection harvests were plaqued onto Madin-Darby canine kidney (MDCK) cell monolayers (Snyder et al., J. Virol. 61: 2857-2863 (1987)), and virus titrations and plaque assays were carried out in MDCK cell monolayers (Murphy et al., Infect. Immun. 37: 1119-1126

(1982)), which publications are incorp rated herein by reference.

The pTZ18U plasmid DNA with the full-length PB2 gene from the A/Ann Arbor/6/60 (A/AA/6/60) virus (pT3AAwtPB2) generated as described in Example 1 was used as the template for site-directed mutagenesis for generating the mt 112 plasmid. The pTZ18U plasmid DNA with the full-length PB2 gene from the A/Ann Arbor/6/60 (A/AA/6/60) virus bearing a single nucleotide (nt) substitution at nt 821 (pT3AAmt265) generated as described in Example 1 was used as the template for site-directed mutagenesis in generating the double mt 265+112 plasmid. Plasmid DNA generated after successful mutagenesis was digested overnight with Hga I (New England Biolabs, Beverly, MA) and filled in with Klenow fragment (New England Biolabs, Beverly, MA) before use as a template in transcription reactions.

For site-directed mutagenesis, the procedure outlined for the Muta-gene phagemid in vitro mutagenesis kit (BioRad Laboratories, Richmond, CA) was followed. The oligonucleotide designed for mutagenesis at residue 112 was as follows: nt 352 5' GTTCATTATTCAAAAATC 3' [SEQ ID No. 4]. The nucleotide sequence of the recovered mutagenized cDNA with the mutation at residue 112 was confirmed to differ from the A/AA/6/60 wt PB2 plasmid only at nt 361 (underlined in mutagenesis oligonucleotide above) where a T replaced a C, predicting a substitution of serine for a proline. The cDNA with mutations at residues 265 and 112 differed from the A/AA/6/60 wt PB2 plasmid at nt 361 as above and, as expected at nt 821 where a G replaced an A to predict an amino acid substitution of serine for asparagine.

Ribonucleoprotein (RNP) was prepared from virus purified from 500 embryonated eggs inoculated with the avian influenza A/Duck/Oklahoma/4/77 as generally described in Parvin et al., J. Virol. 63: 5142-5152 (1989).

For the transfection system, T3 polymeras transcription r acti ns, incubated at 37°C for 55 min and treated with DNas for 5 minutes, were transfected using DEAE-dextran into primary chick kidney cells infected with the PB2

SGR helper virus as described in Example 2. Transfections were incubated at 32°C overnight and were harvested 22 to 24 hr later.

PB2 transfectant viruses that derived the PB2 gene from the plasmid were identified as described in Example 4. Plaque progeny underwent a total of three plaque-to-plaque passages on MDCK cell monolayers, were amplified once in MDCK cells and then in the allantoic cavity of embryonated eggs.

For sequence analysis, the PB2 gene from transfectant virus was amplified as described above in Example 4 in two separate reactions as overlapping segments (nt 1 to 1240 and 1141 to 2341) from virion RNA by polymerase chain reaction (PCR) with avian myeloblastosis virus reverse transcriptase to synthesize at the first strand and subsequently conventional methods of PCR amplification were used. The nucleotide sequences of the PCR products were determined with the Circum Vent thermal cycler dideoxy DNA sequencing kit (New England Biolabs, Beverly, MA) with 35S-dATP (Amersham Corp., Arlington Heights, IL). The entire length of the gene was sequenced using 13 oligonucleotide primers that were synthesized in an Applied Biosystems 380A DNA synthesizer.

The rescue of the A/AA/6/60 wt PB2 gene and a mutant derivative of the gene with a mutation at amino acid 265 are described in Examples 2-4. This mutation at amino acid 265 conferred the temperature sensitivity and attenuation phenotypes on the transfectant virus. To expand the menu of ts mutation sites and to determine whether a ts mutation identified in a different influenza A virus would specify the ts phenotype when inserted into the A/AA/6/60 wt PB2 gene by reverse genetics, a mutation was selected that is present in three independently derived influenza A/Udorn/72 ts mutants ICRC303AA, SP374AB and SP959CC, which each had a substitution at nt 361 coding for an amino acid substitution of a serine for a proline at residue 112 (Lawson et al., Virology 191:506-510 (1992)). Of 19 plaques, three plaques were isolated with the AA PB2 gene, one was selected (designated A/LA/2/87 AA mt 112 PB2 clone 12D1), plaque purified and confirmed by

nucleotide sequencing t differ from the A/LA/2/87 AA PB2 gene only at amino acid 112.

The two mutations (at amino acids 265 and 112) were combined to confirm that the transfection-rescue system would operate efficiently to rescue "double mutant" viruses. Of 24 plaques, eight transfectant virus plaques were isolated with the AA PB2 gene, one was selected (designated A/LA/2/97 AA mt 265+112 PB2 clone 6A1), plaque purified and confirmed by nucleotide sequencing to differ from the AA wt PB2 gene at amino acids 265 and 112.

Example 9
Temperature Sensitivity of Double Mutant PB2 Transfectant Viruses

This Example demonstrates that the double mutant PB2 transfectant virus is more temperature sensitive than the single mutant PB2 transfectant viruses bearing either mutation alone.

The efficiency of plaque formation (EOP) at different temperatures of the PB2 transfectant viruses bearing one or two mutations in the PB2 gene was compared with each other and that of the A/LA/2/87 AA wt PB2 transfectant virus The EOP of the mutants was determined at 32°, 37°, 38°, 39° and 40°C in two to four separate experiments with MDCK cell monolayers as described above. Virus titers were expressed as mean $\log_{10}$ PFU/ml for the experiments. The mt PB2 transfectant viruses bearing mutations at amino acid 265 or 112 alone showed a restriction of replication at 39°C and 40°C, with each demonstrating a shut-off temperature of 38°C. The A/LA/2/87 AA mt 265+112 PB2 transfectant virus was completely restricted in replication at temperatures 39° and 40°C, and displayed a shut-off temperature of 38°C. Thus, the addition of a second mutation to th PB2 gene led to further restriction of replication at elevated temperatures over the

level of restriction imposed by a single mutation in this gene.

Table 5

Comparison of the ts phenotype of PB2 transfectant viruses bearing one or two mutations in the PB2 gene

A/LA/2/87 AA PB2 transfectant virus	Titer at 32°C[a]	Log_{10} reduction in pfu/ml at indicated temperature compared with titer at permissive temperature (32°C)[a]				Shutoff temperature (°C)[b]
		37°C	38°C	39°C	40°C	
wt	6.6	0.6	0.7	1.4	4.1	40
mt 265	6.3	0.5	1.0	3.4	5.0	39
mt 112	6.5	1.0	1.2	2.2	5.1	39
mt 265 + 112	6.1	1.0	2.5	≥5.4	≥5.4	38

[a] Titers expressed are the mean of two or four experiments

[b] The shutoff temperature, indicated by bold numbers, is defined as the lowest restrictive temperature at which there is a ≥2 log_{10} reduction in titer from the titer at the permissive temperature.

Double mutants A/LA/2/87 AA mt 265+556 PB2 and A/LA/2/87 AA mt 265+658 PB2 were also rescued according to the above protocols. The results of temperature sensitivity testing with these double mutants, shown in Table 6, indicated that A/LA/2/87 AA mt 265+556 PB2 displayed a shutoff temperature of 38°C, compared to 39°C and 40°C of the corresponding single mt viruses, and A/LA/2/87 AA mt 265+658 PB2 displayed a shutoff temperature of 39°C, compared to 39 and 40°C of the corresponding single mt viruses. However, the AA mt 265+658 PB2 transfectant virus differed from its two corresponding single mt transfectant viruses in being completely restricted in plaque formation at 39°C. Although there were slight differences in mean log_{10} reductions in titer of mt PB2 transfectant viruses from experiment to experiment (comparing Tables 5 and 6), the AA single mt transfectant viruses as a group were more restricted than the AA wt PB2 transfectant virus at 40°C and the AA double mt transfectant viruses were more restricted as a gr up than the AA singl mt PB2 transfectant viruses at 39°C. This indicat s

that there is an incremental increase in the level of temperature sensitivity as additional mutations are acquired.

Table 6

PB2 transfectant viruses bearing two mutations are more temperature sensitive than transfectant viruses bearing single mutations

A/LA/2/87 AA PB2 transfectant virus	Titer at 32°C[a]	Log_{10} reduction in titer (pfu/ml) at indicated temperature compared to titer at permissive temperature (32°C)					Shutoff temperature[b]
		36°C	37°C	38°C	39°C	40°C	
wt	6.5	-0.2	-0.2	0.1	1.2	2.4	40°
mt 112	6.5	0.2	0.3	0.6	1.7	≥5.8c	40°
mt 265	6.5	0.2	0.4	0.8	1.9	≥5.8c	40°
mt 556	6.1	0.3	0.9	1.5	4.5	≥5.4c	39°
mt 658	6.1	0.2	0.4	0.8	2.9	≥5.4c	39°
mt 265 + 112	6.4	0.4	0.9	2.1	≥5.7c	≥5.7c	38°
mt 265 + 556	6.1	0.6	1.1	2.0	≥5.4c	≥5.4c	38°
mt 265 + 658	6.1	0.3	0.9	1.4	≥5.4c	≥5.4c	39°

[a] Titers expressed are the mean of two experiments

[b] The shutoff temperature, indicated by bold numbers, is defined as the lowest restrictive temperature at which there is a ≥2 log_{10} reduction in titer from the titer at the permissive temperature.

Example 10

Double Mutant PB2 Transfectant Viruses Exhibit Restricted Replication In Upper and Lower Respiratory Tracts of Animals

This Example demonstrates that a PB2 transfectant virus bearing two mutations in the PB2 gene is more restricted in the upper and lower respiratory tracts of hamsters than PB2 transfectant viruses bearing either mutation alone.

The level of replication in the respiratory tract and the protective efficacy of transfectant viruses in hamsters were compared. The levels of virus replication in the respiratory tract of hamsters of the A/LA/2/87 AA wt, mt 112, mt 265, and mt 265+112 PB2 transfectant viruses were determined, as were the abilities of the A/LA/2/87 AA wt and

mt 265+112 PB2 transfectant viruses to induce an antibody response and protect animals from challeng with a wt A/LA/2/87 virus.

A $10^{5.0}$ $TCID_{50}$ inoculum of the indicated virus was administered intranasally in 0.1 ml to anesthetized five-week old Golden Syrian hamsters on day 0. On day 28, the animals were bled and similarly challenged with $10^{5.0}$ $TCID_{50}$ of wt A/LA/2/87 virus. Animals were sacrificed one day after virus challenge, lungs and nasal turbinates were removed and homogenized and virus titers in respective tissues were determined. Titers of hemagglutination-inhibiting antibodies in sera were measured against the A/LA/2/87 wt virus as described in Example 7 and Clements et al., supra.

The levels of replication of the A/LA/2/87 AA wt, mt 112, mt 265, and mt 265+112 PB2 transfectant viruses in the hamsters are shown in Fig. 5. The AA mt 112 and AA mt 265 PB2 transfectant viruses differed in sequence from the AA wt PB2 transfectant virus by only one amino acid and the AA mt 265+112 PB2 transfectant virus differed in sequence from each of the two single mutant viruses by only one amino acid. Therefore, the effects of the single mutations and the addition of a second mutation on replication in vivo were directly assessed. Because the body temperatures of hamsters is 38.7°C ± 0.3°C, and the shut-off temperature of the mutant PB2 transfectant viruses were 38° and 39°C, a mild to moderate degree of attenuation of these viruses was expected in the hamsters. The A/LA/2/87 mt 265 and mt 112 PB2 transfectant viruses replicated in the upper respiratory tract (nasal turbinates) to levels similar to the AA wt PB2 transfectant virus, but they displayed lower levels of replication in the lower respiratory tract (lungs). The A/LA/2/87 AA mt 265+112 PB2 transfectant virus, on the other hand, replicated to lower titers in the nasal turbinates ($p < 0.0001$ vs AA wt and mt 112 and mt 265 PB2 transfectant viruses) and was undetectable in the lungs ($p < 0.01$ vs. AA wt and AA mt 265 PB2 transfectant viruses). It was unexpectedly observed that the A/LA/2/87 AAwtPB2 transfectant virus (with the AA PB2 g ne) was more restricted in replication in the lungs of hamsters than the

A/LA/2/87 wt virus (with the A/LA/2/87 PB2 gene), an observation that was attributed to a gene constellation effect. The A/LA/2/87 wt virus replicates to titers up to 6 $\log_{10}$ $TCID_{50}$/g in the lungs of hamsters (Table 3) whereas the A/LA/2/87 AA wt PB2 transfectant virus is more restricted (Fig. 5B). The evaluation of level of replication in hamsters clearly indicates that the double mutant transfectant virus was more restricted than either single mutant transfectant virus alone and that the virus was completely restricted in the lower respiratory tract.

Table 7

PB2 transfectant viruses bearing two mutations are more restricted in the upper and lower respiratory tract of hamsters than transfectant viruses bearing single mutations

A/LA/2/87 AA PB2 transfectant virus administered[a]	Shut-off temp (°C)[b]	Virus titer (log_{10} $TCID_{50}$/g) in indicated tissue on indicated days[c,d]					
		Nasal Turbinates[e]			Lungs[e]		
		Day 2	Day 3	Day 4	Day 2	Day 3	Day 4
wt	40°	5.8 ± 0.2	5.5 ± 0.2	5.4 ± 0.2	1.9 ± 0.2	2.6 ± 0.7	2.7 ± 0.6
mt 112	40°	4.9 ± 0.3	5.7 ± 0.2	5.1 ± 0.1	1.7 ± 0.1	3.9 ± 0.6	1.8 ± 0.2
mt 265	40°	5.4 ± 0.3	5.4 ± 0.2	5.2 ± 0.3	1.6 ± 0.1	2.2 ± 0.4	1.6 ± 0.1
mt 556	39°	5.0 ± 0.1	5.3 ± 0.1	5.0 ± 0.2	≤1.5 ± 0.0	≤1.5 ± 0.0	≤1.5 ± 0.0
mt 658	39°	5.5 ± 0.3	5.5 ± 0.1	4.8 ± 0.2	2.5 ± 0.5	2.4 ± 0.6	1.7 ± 0.2
mt 265 + 112	38°	3.8 ± 0.5	4.2 ± 0.3	3.7 ± 0.3	≤1.5 ± 0.0	≤1.5 ± 0.0	≤1.5 ± 0.0
mt 265 + 556	38°	4.3 ± 0.2	4.0 ± 0.3	4.0 ± 0.2	≤1.5 ± 0.0	≤1.5 ± 0.0	≤1.5 ± 0.0
mt 265 + 658	39°	4.7 ± 0.5	4.8 ± 0.5	4.9 ± 0.0	≤1.5 ± 0.0	≤1.5 ± 0.0	≤1.5 ± 0.0

[a] Viruses were administered in doses of $10^{5.0}$ $TCID_{50}$ in a 0.1 ml inoculum to anesthetized Golden Syrian hamsters.

[b] Shut-off temperatures from in vitro tests of efficiency of plaque formation.

[c] Five animals were tested on days 2 and 3 for mt 112, mt 265, mt 556 and mt 658 and six animals per day for the remaining viruses.

[d] Titers are expressed as mean log_{10} $TCID_{50}$/g ± SE; the lower limit of detection of virus in tissues is log_{10} 1.5 $TCID_{50}$/g.

[e] Virus titers in 5% wt/vol suspensions of nasal turbines and 10% wt/vol suspensions of lung tissue were determined in MDCK tissue culture.

The PB2 transfectant virus bearing two mutations in the PB2 gene was shown to be immunogenic and to protect hamsters from wild-type virus challenge. The ability of the double mutant PB2 transfectant virus to induce a significant antibody response and to protect from wild-type virus challenge, despite its restriction in replication, are important properties for a vaccine candidate. The A/LA/2/87 AA mt 265 PB2 transfectant virus was shown in Example 7 above to be immunogenic and able to protect from wild-type virus challenge, so only the double mutant PB2 transfectant virus and appropriate controls were included in this analysis. As shown in Table 8, the challenge virus, influenza A/LA/2/87 wt virus was administered 28 days after immunization with the AA wt and mt 265+112 PB2 transfectant viruses or influenza B/AA/1/86 virus. Lungs and nasal turbinates were harvested one day post infection because preliminary experiments indicated that peak titers following infection with the wild-type challenge virus were achieved one day post-inoculation.

Table 8

A PB2 transfectant virus bearing two mutations is immunogenic and protects hamsters from wild-type virus challenge[a]

Immunizing Virus	No. of animals	Response to immunizing virus: Serum HAI Ab titer (recip mean $log_2 \pm SE$)	Response to wt virus challenge (log_{10} $TCID_{50}$/g) in indicated tissue[b]: Lungs	Nasal turbinates
B/Ann Arbor/1/86	6	<2.0 ± 0	6.5 ± 0.4	6.3 ± 0.2
AA wt PB2 transfectant	6	6.7 ± 0.7	2.2 ± 0.7	2.7 ± 0.5
AA mt 265 + 112 transfectant	6	5.5 ± 0.2	2.1 ± 0.6	2.8 ± 0.6

[a] $10^{5.0}$ $TCID_{50}$ of indicated virus was administered intranasally in a 0.1 ml inoculum to anesthetized 5 wk old Golden Syrian hamsters on day 0. On day 28, the animals were bled and similarly challenged with $10^{5.0}$ $TCID_{50}$ of wt A/LA/2/87 virus.

[b] Animals were sacrificed one day after virus challenge, lungs and nasal turbinates were removed and homogenized and virus titers in respective tissues were determined. Titers are expressed as mean log_{10} $TCID_{50}$/g ± SE.

Despite the attenuation of the double mt PB2 transfectant virus in hamsters (Fig. 5), the virus was highly immunogenic and induced a high level of protection against wild-type virus challenge which was similar to that induced by the A/LA/2/87 AA wt PB2 transfectant virus, thereby confirming feasibility of its use as a vaccine.

Example 11

The Double Mutant PB2 Transfectant Virus Displays Greater Genetic Stability After Prolonged Replication

The level of gen tic stability of the transfectant viruses was evaluated by testing for l ss f the ts ph notype of the viruses after prolonged replication (14 days) in vivo

in immunocompromised hosts (nude mice). Fourteen days of replication was selected as the time point becaus data from human influenza A live virus vaccine trials suggest that live attenuated influenza virus vaccine can replicate for up to 14 days in fully susceptible seronegative subjects and therefore the genetic stability of the ts phenotype, a marker for attenuation of a vaccine virus, should be evaluated after 14 days of replication in vivo.

The A/LA/2/87 AA wt, mt 112, mt 265 and mt 265+112 PB2 transfectant viruses were inoculated intranasally into 4 to 6 week old nude Balb/C or NIH Swiss mice and were allowed to replicate for 14 days. The animals were then sacrificed and lungs and nasal turbinates removed and homogenized. Each specimen (lungs and nasal turbinates) was cultured for virus and each isolate was then tested for its efficiency of plaque formation at 32 and 39.5°C to determine the genetic stability of the ts phenotype of viruses isolated from animals after prolonged replication in vivo.

The results, shown in Table 9, indicated that the A/LA/2/87 AA PB2 transfectant viruses could not be recovered from the lungs of nude mice after 14 days, indicating a level of restriction of virus replication possibly imposed by the presence of the AA PB2 gene in an influenza A/LA/2/87 virus background. While virus could be recovered from the nasal turbinates of a moderate to high percentage (40% and 80%, respectively) of nude mice inoculated with the AA mt 112 and mt 265 PB2 transfectant viruses, virus could be recovered from only a few (10 of 115, or 9%) mice inoculated with the A/LA/2/87 AA mt 265+112 PB2 transfectant virus (Table 9). This further supports the observations in hamsters that the double mutant transfectant virus was highly attenuated in rodents. Each virus isolate was tested for efficiency of plaque formation at 32 and 39.5°C to determine the number of virus isolates with partial loss of the ts phenotype. The double mutant PB2 transfectant virus was significantly less likely than the single mutant PB2 transfectant viruses to lose the ts phenotype after prolonged replication in the upper respiratory tract f the nude mice.

Table 9

The ts phenotype of a PB2 transfectant virus bearing two mutations is more stable after prolonged replication in the respiratory tracts of nude mice than transfectant viruses with either mutation alone

A/LA/2/87 AA PB2 transfectant virus administered	No. of animals inoculated[a]	No. of mice from which virus isolates were obtained[b]	% of virus isolates with partial loss of ts phenotype[c]	
mt 265	24	21 (80%)	43	
mt 112	55	19 (40%)	42	d
mt 265 + 112	115	10 (9%)	0[e]	

[a] $10^{5.0}$ $TCID_{50}$ of virus of 0.1 ml was administered intranasally to anesthetized nude mice.

[b] Fourteen days after inoculation, the lungs and nasal turbinates of the mice were harvested and homogenized. Isolates were only obtained from the nasal turbinates.

[c] Virus isolates in MDCK cells incubated at 32°C were tested for efficiency of plaque formation at 32°C and 39.5°C. Isolates with a partial loss of ts phenotype replicated at 39.5°C to a titer 100-fold or more than that of sister isolates that failed to plaque at 39.5°C.

[d] $p = 0.05$ (Fisher's exact test)

[e] A single plaque was present at the 10^{-2} dilution at 39.5°C.

One isolate from a nude mouse that received an intranasal injection of the A/LA/2/87 AA mt 265+112 PB2 transfectant virus yielded a single plaque at the 10^{-2} dilution at 39.5°C. The virus was amplified at 32°C and was confirmed to be ts+. Nucleotide sequence analysis across the two mutation sites revealed that reversion had not taken place at either site, indicating that the basis for the reversion of the ts phenotype was an intragenic or extragenic suppressor mutation.

All publications mentioned in this specification are indicative of the level of skill of those skill d in the art

to which this invention pertains. All publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated herein by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: The Government of the United States of America, as represented by the Secretary of the Department of Health and Human Services
(B) STREET: Box OTT
(C) CITY: Bethesda
(D) STATE: Maryland
(E) COUNTRY: United States of America
(F) POSTAL CODE (ZIP): 20892
(G) TELEPHONE: (301) 496-7056
(H) TELEFAX: (301) 402-0220
(I) TELEX:

(ii) TITLE OF INVENTION: METHOD FOR GENERATING INFLUENZA A VIRUSES BEARING ATTENUATING MUTATIONS IN INTERNAL PROTEIN GENES

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO
(B) FILING DATE:
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/123,993
(B) FILING DATE: 20-SEP-1993

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Parmelee, Steven W.
(B) REGISTRATION NUMBER: 31,990
(C) REFERENCE/DOCKET NUMBER: 15280-163-1

(viii) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 206-467-9600
(B) TELEFAX: 415-543-5043

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTTCATTATT CAAAAATC 18

WHAT IS CLAIMED IS:

1. A method for producing an attenuated influenza A virus comprising the steps of:

(a) transfecting into cultured non-mammalian cells an isolated gene which comprises an RNA template encoding an internal protein of human influenza A virus, said internal protein when present in an influenza virus causing attenuated growth in mammalian cells, wherein said cultured non-mammalian cells are infected with a strain of influenza A virus incapable of growing in mammalian cells and that encodes an allele of the internal protein, said allele being incapable of supporting growth in mammalian cells;

(b) cultivating the transfected cells under culture conditions conducive to propagation of influenza virus; and

(c) isolating from the transfected cells influenza A virus containing the transfected internal protein gene and which virus is capable of attenuated growth on mammalian cells.

2. The method of claim 1, wherein the transfected internal protein gene is the polymerase basic protein 1 (PB1) gene, the polymerase basic protein 2 (PB2) gene, or the polymerase acidic protein (PA) gene.

3. The method of claim 2, wherein the attenuated growth of the isolated virus results from a temperature-sensitive mutation comprising an amino acid substitution in the PB1, PB2 or PA gene, respectively.

4. The method of claim 3, wherein the amino acid substitution is in the PB2 gene at position 65, 100, 112, 174, 265, 298, 310, 386, 391, 417, 512, 556, or 658.

5. An attenuated influenza A virus produced by the method of claim 1.

6. The attenuated influenza virus of claim 5, wherein the transfected internal protein gene is PB2 and the attenuated growth of the isolated virus results from a temperature-sensitive mutation comprising an amino acid substitution in the PB2 gene at position 112, 265, 556 or 658.

7. The attenuated influenza virus of claim 6, wherein the PB2 gene comprises temperature-sensitive mutations encoding amino acid substitutions at residues 112 and 265, 265 and 556, or 265 and 658.

8. The attenuated influenza virus of claim 5, wherein the transfected internal protein gene is PB2 and further comprising a temperature-sensitive mutation in the PA gene.

9. The method of claim 1, wherein the influenza A virus infecting the non-mammalian cells and which is not capable of growth in mammalian cells contains a PB2 protein having the amino acid glutamic acid at position 627.

10. A method for recovering a hemagglutinin (HA) or neuraminidase (NA) gene from a wild-type strain of influenza A virus to produce an attenuated influenza A virus having the HA or NA wild-type gene comprising the steps of:

(a) co-infecting a culture of mammalian or avian cells with the wild-type strain and the attenuated influenza virus produced by the method of claim 1;

(b) recovering reassortant viruses from the infected cells; and

(c) identifying attenuated virus from among the reassortant viruses that have incorporated the HA and/or NA gene of the wild-type virus.

11. The method of claim 10, wherein the HA and NA genes of the wild-type strain are incorporated into the attenuated virus identified in step (c).

12. The attenuated influenza A virus produced by the method of claim 11 which expresses the wild-type HA or NA gene.

13. A method for vaccinating a host against the wild-type influenza A strain of claim 10 which comprises inoculating the host with the attenuated virus produced by the method of claim 10.

14. A method for producing an attenuated influenza A virus vaccine comprising the steps of:

(a) transfecting a cell line with an RNA molecule transcribed *in vitro* from DNA that encodes an internal protein of human influenza A virus, said internal protein causing attenuated growth in host cells, wherein the transfected cells are infected with a first strain of influenza A virus that encodes an allele of the internal protein that permits efficient rescue of the transfected RNA into virus; and

(b) isolating from the transfected cells an attenuated virus which contains the transfected internal protein gene and which is capable of attenuated growth in host cells.

15. The method of claim 14, wherein said first strain of influenza A virus is a wild-type isolate of human influenza A.

16. The method of claim 14, wherein said internal protein is PB2 and said DNA encoding PB2 contains at least two ts mutations that result in said attenuated growth.

17. The method of claim 16, further comprising the steps of co-infecting a cell line with said attenuated influenza A virus and with a virus containing a PA gene that contains a ts attenuating mutation, and isolating from the co-infected cells a reassortant virus that contains both ts mutations in the PB2 gene and the ts mutation in the PA gene.

18. A method for attenuating a wild-type human influenza A virus, comprising the steps of:

(a) co-infecting a culture of mammalian cells with the attenuated virus of claim 14 and the wild-type strain;

(b) recovering reassortant virus from the culture of infected cells; and

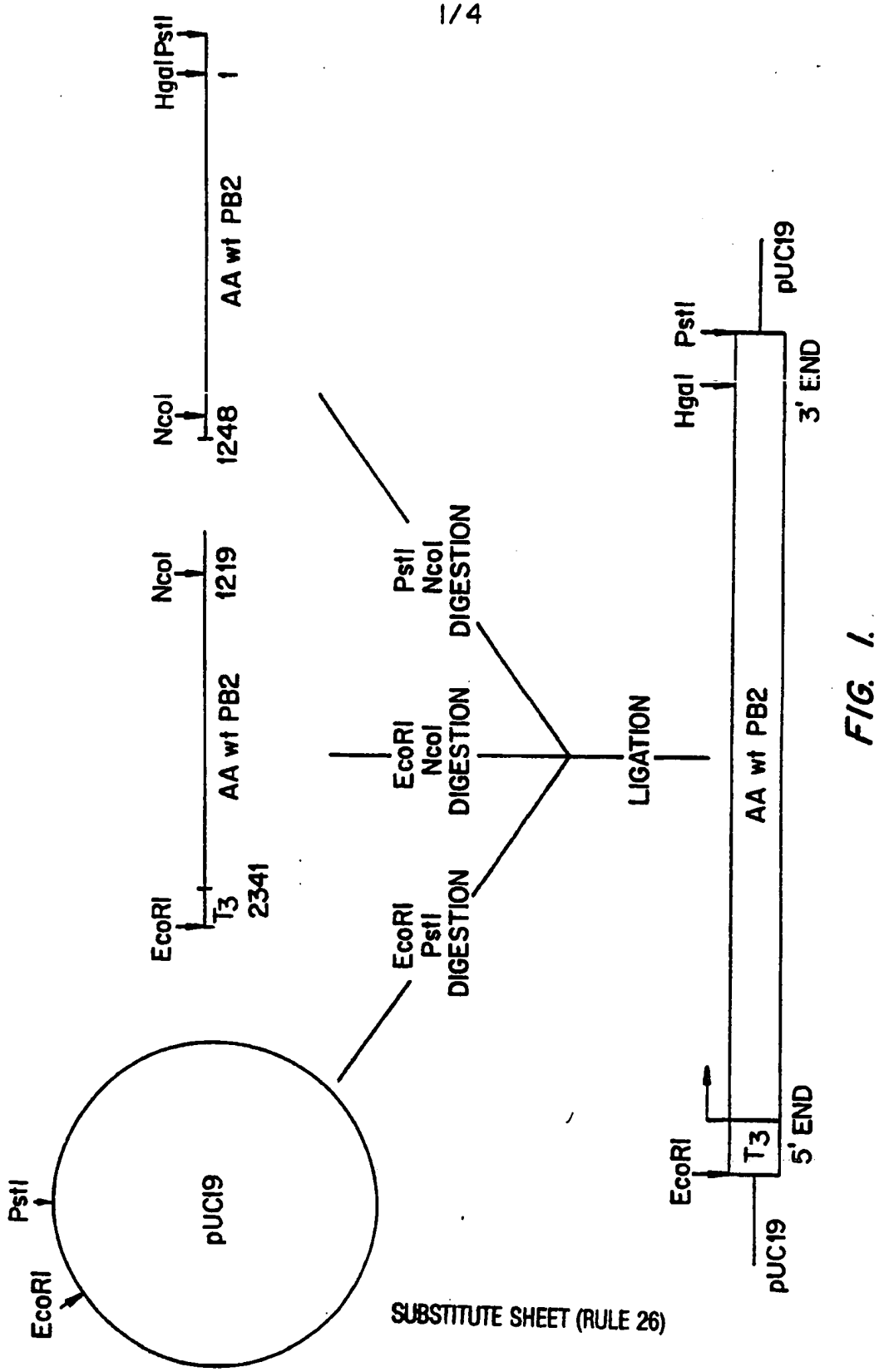
(c) identifying attenuated reassortant virus that has incorporated an HA or NA gene of the wild-type virus.

19. An attenuated reassortant virus produced by the method of claim 18.

20. A method for vaccinating a host against a wild-type influenza A virus which comprises inoculating said host with the attenuated reassortant virus of claim 19 in a manner sufficient to allow attenuated growth of the virus in the host for a time sufficient to produce a protective immune response against the influenza virus.

21. The vaccination method of claim 20, wherein both the HA and NA genes of the wild-type influenza A strain are incorporated into the attenuated reassortant virus.

22. The vaccination method of claim 21, wherein the attenuated virus is administered to mucosal tissue of the host by drops or aerosol.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LIGATION
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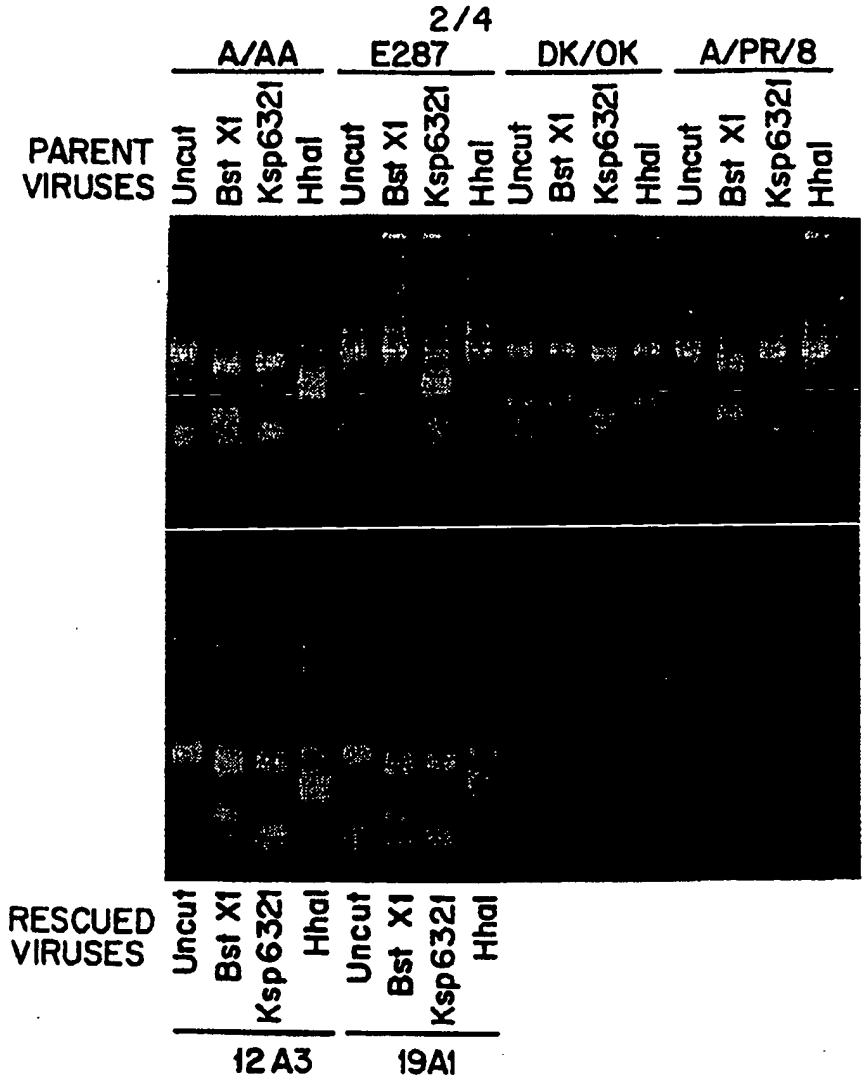
FIG. 1.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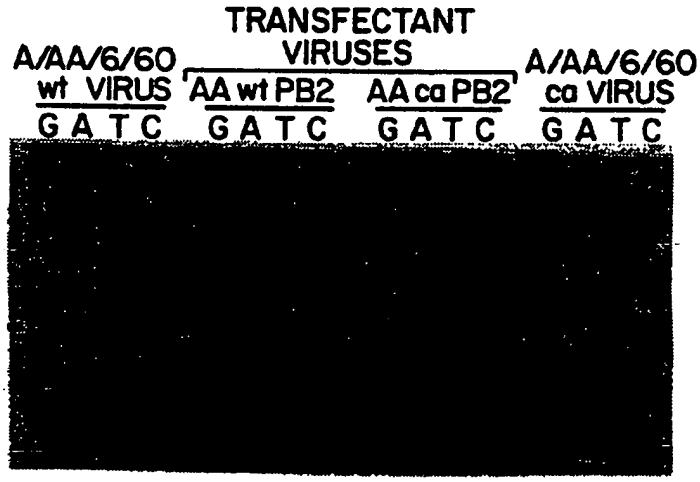
19A1

FIG. 2.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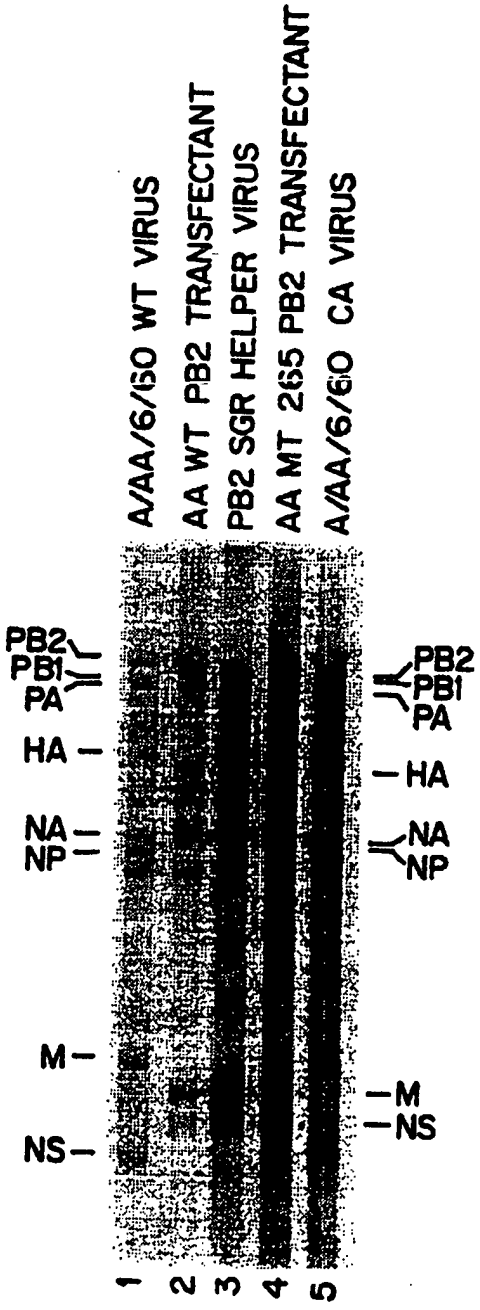
G A T C
G A T C
G A T C

FIG. 4.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PB2 SGR HELPER VIRUS
AA MT 265 PB2 TRANSFECTANT
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HA
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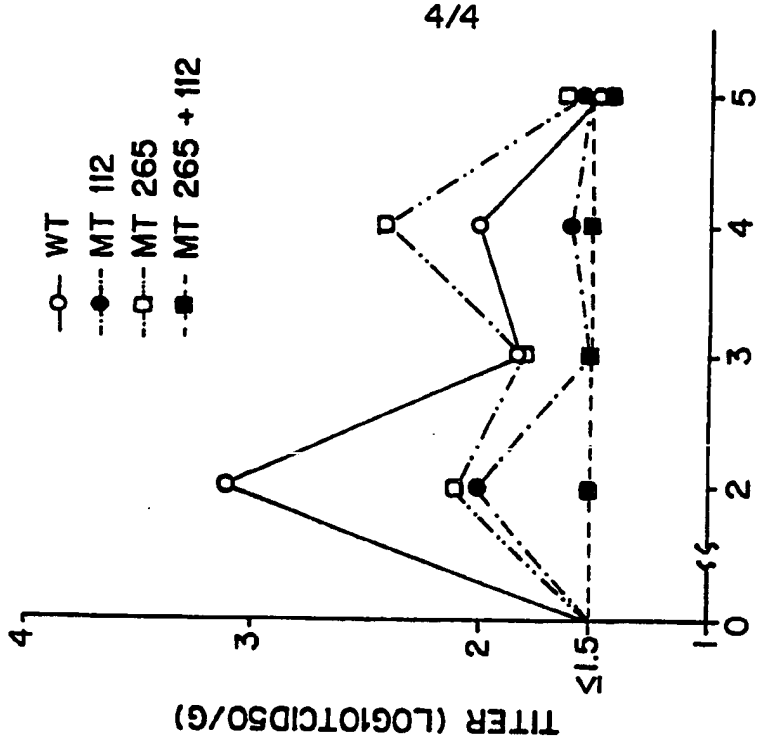
NP
M
NS
1
2
3
4
5

FIG. 3.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MT 112
MT 265
MT 265 + 112
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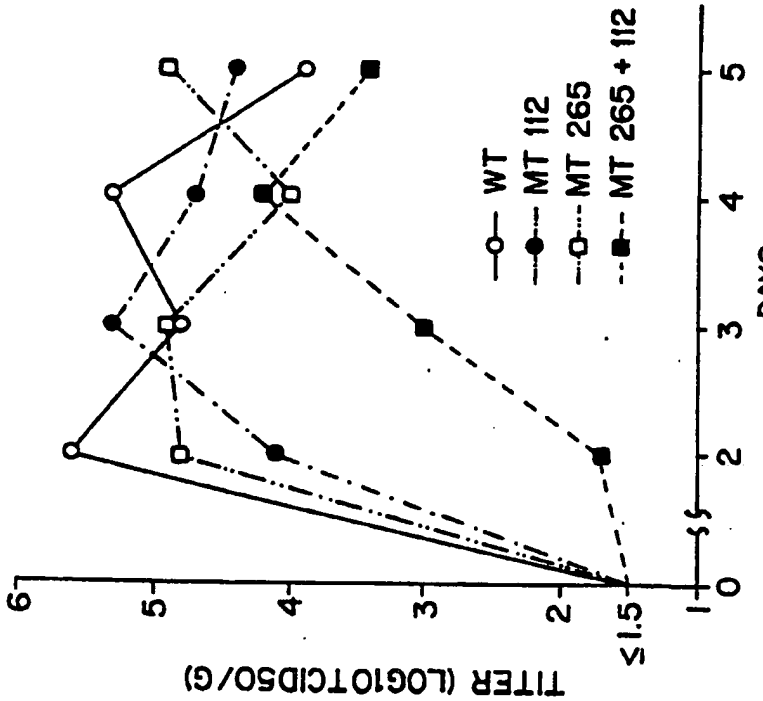
DAYS

FIG. 5B.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MT 265
MT 265 + 112
TITER (LOG10TCID50/G)
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4
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1
2
3
4
5
DAYS

FIG. 5A.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/10597

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 A61K39/145 C12N9/10 C12N7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VIROLOGY, vol.191, 1992 pages 506 - 510 C. LAWSON ET AL. 'Nucleotide sequence changes in the polymerase basic protein 2 gene of temperature-sensitive mutants of influenza A virus' see the whole document	1-7, 9-16, 18-22
Y	---	8,17
Y	PROC. NATL. ACAD. SCI. USA, vol.83, 1986 pages 2709 - 2713 M. KRYSTAL ET AL. 'Expression of three influenza virus polymerase proteins in a single cell allows growth complementation of virla mutants' see page 1, column 2, paragraph 2	8,17

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
22 February 1995	28.02.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Skelly, J

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	J. VIROL., vol.67, no.12, 1993 pages 7223 - 7228 E. K. SUBBARAO ET AL. 'Rescue of an influenza A virus wild-type PB2 gene and a mutant bearing a site-specific temperature-sensitive and attenuating mutation' see the whole document	1-22
X,P	WO,A,93 21306 (THE MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK) 28 October 1993 see page 21, line 3 - line 24	1-22
A	J. CLIN. MICRO., vol.30, no.3, 1992 pages 655 - 662 M. L. CLEMENTS ET AL. 'Use of single gene reassortant viruses to study the role of Avian influenza virus genes in attenuation of wild-type human influenza virus for squirrel monkeys and adult human volunteers'	
A	VIROLOGY, vol.186, 1992 pages 795 - 797 A. KLIMOV ET AL. 'Sequence changes in the live attenuated cold-adapted variants of Influenza A/Leningrad/134/57 (H2N2) virus'	
A	J. VIROL., vol.62, no.2, 1988 pages 488 - 495 M. SNYDER ET AL. 'Four viral genes independently contribute to attenuation of live influenza A/Ann Arbor/6/60 (H2N2) cold-adapted reassortant virus vaccines'	
A	PROC. NATL. ACAD. SCI. USA, vol.87, 1990 pages 3802 - 3805 M. ENAMI ET AL. 'Introduction of site-specific mutations into the genome of influenza virus'	

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INTE[redacted]ONAL SEARCH REPORT

[illegible]ation on patent family members

[illegible]nal Application No
PCT/US 94/10597

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9321306	28-10-93	AU-B-	4289393	18-11-93
		CN-A-	1082606	23-02-94
		EP-A-	0636172	01-02-95